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NF-κB is Involved in the Induction of the Rat Hepatic α1-Acid Glycoprotein Gene by Phenobarbital

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Phenobarbital, a classical inducer of the drug-metabolizing cytochrome P450 genes, induces α1-acid glycoprotein gene expression through a PB-responsive element (PBRE) located at position −142 to −126 from the transcriptional start site. The aim of this study was to investigate nuclear protein binding to the PBRE sequence after PB treatment. Cycloheximide treatment showed that *de novo* protein synthesis was not required for PB to induce AGP gene expression, pointing to post-translational modifications. Studies of the DNA–protein complex with the PBRE showed that phosphorylation status is a key regulator of the binding capacity of transactivating proteins involved in PB transcriptional activation. This DNA–protein complex, analyzed by southwestern blotting and UV cross-linking, involves three nuclear factors with molecular weights of 43, 52, and 65 kDa. Supershift and competition experiments showed that the 43-kDa factor can be related to C/EBPα and the 52- and 65-kDa factors to the two subunits of NF-κB. © 1999 Academic Press

Phenobarbital (PB) has long been known to induce the expression of a large number of liver-specific genes, especially those encoding drug- and steroid-metabolizing enzymes (1, 2). The main enzymes responsive to PB are cytochromes P450 2B1 and 2B2 (CYP2B1/2) (3-5); we recently found that PB increased the expression of the α1-acid glycoprotein (AGP) gene both *in vivo* and *in vitro* (6, 7), AGP being a member of the acute-phase protein family (8, 9). Although most of these inductive effects are of a transcriptional nature (6, 10), the process by which PB activates nuclear gene transcription remains unknown; both receptor-dependent and -independent mechanisms have been considered (11). A steroid receptor is thought to participate indirectly in *CYP* gene induction by PB in hepatoma cells (12), in which PB treatment can lead to accumulation of an endogenous steroid-like compound which then binds to its specific receptor to form a complex that directly activates CYP gene expression. The PB receptor protein has not yet been identified.

As PB mainly induces gene expression through transcriptional activation, many teams have attempted to identify cis-acting elements of the *CYP*2B1/2 gene promoter. Several responsive elements have been characterized in proximal regions, with positive elements at positions −98 to −69, (13); −199 to −183 and −72 to −31, (14, 15); −61 to −45 and −116 to −129, (16); −64 to −45 and −138 to −119, (17); −103 to −66, (18); and −89 to −73 (19); and negative elements at position −127 to −160, (20). Functional analysis of these elements by using DNA-asialoglycoprotein complexes to target the DNA to the liver injection into the whole animal has been proposed as a model for the modulation of the PB-responsive minimal promoter (21). Another responsive element (an enhancer) has been located in the far-upstream region of *CYP*2B1/2 genes, between positions −2155 and −2317 (22-25). Studies with transgenic mice have shown that an important PB genetic element resides between 1.7 and 2.3 bp upstream of the *CYP*2B2 transcriptional start site (26).

As regards the AGP gene, we have previously demonstrated in transfection experiments with primary rat hepatocytes that a 17-bp sequence, mapping to position −142 to −126 from the transcription start site, can confer PB inducibility to a heterologous gene (27). This sequence is similar to that described in *B. megaterium* and rat *CYP*2B1/2 genes (19), and designated the "Barbie box" by Shaw and Fulco (28). Furthermore, gel retardation assays have shown that the Barbie box sequence of the *CYP*2B1/2 and AGP genes bind proteins from rat liver nuclei in a PB-dependent manner *in vitro* (19, 27). The aim of this study was to determine the nature of the proteins binding to the Barbie box

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Abbreviations used: AGP, Alpha-1-acid glycoprotein; C/EBP, CAAT enhancer binding protein; CYP, Cytochrome P450; PB, Phenobarbital; PBRE, Phenobarbital responsive element; SD, Sprague Dawley.

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sequence of the AGP gene and leading to its activation after PB treatment.

MATERIALS AND METHODS

Chemicals. Lyophilized sodium phenobarbital (Gardenal for subcutaneous or intramuscular injection) was from Specia (Paris, France). Calf intestinal phosphatase was from Boehringer Mannheim (Paris, France). Cycloheximide was from Sigma (St Quentin-Falavier, France). ATP (γ^{32}P) was from Amersham (France). AntiC/EBPα , antiC/EBPβ, antiNF-κB p50/52 and p65 were from Santa Cruz Biotechnology (California).

Animals. Male Sprague Dawley (SD) rats (190-250 g) from Charles Rivers breeders (France) were housed at 21-24°C with a 14 h-10 h light-dark cycle and laboratory chow (UAR, France) and tap water *ad libitum*.

They received daily 75 mg/kg phenobarbital subcutaneously for four days. Control animals received the solvent alone. Liver specimens were collected for the preparation of nuclear extracts, 24 h after the last injection.

Cell culture. Hepatocytes from SD rats were isolated and cultured on Matrigel as previously described (7, 27). Three million hepatocytes were cultured in 10 cm^2 dishes with 3 ml of culture medium. PB 2 mM and/or cycloheximide 10 μM was added to the culture medium 24 h and 48 h after cell adhesion (three hours after seeding).

Sixteen hours after the second addition of PB and/or cycloheximide, total cellular RNA was extracted for northern blot analysis.

Preparation of RNA and northern blot analysis. Total cellular RNA was prepared from primary hepatocyte cultures and analyzed as previously described (27). Briefly, RNA was electrophoresed through 1.2% formaldehyde-agarose gels and blotted onto Hybond N membranes (Amersham, Les Ulis, France) by means of northern capillary transfer, then hybridized to AGP and CYP2B1/2 cDNA probes obtained as previously described (6). Isolated inserts of the recombinant plasmids were labeled by the random priming procedure using (α^{32}P) dCTP as substrate.

Autoradiographs were scanned by means of photometric densitometry (Sebia, Paris France) and results are expressed relative to control values.

Preparation of nuclear extracts. Nuclear extracts were prepared by the procedure described by Andersen et al (29). All solutions subsequently used contained 0.5 mM PMSF, 100 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 0.75 mM spermidine, 0.15 mM spermine and 1 mM β-mercaptoethanol, except for the dialysis buffer which contained 0.2 mM PMSF, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin and 0.5 μg/ml pepstatin.

Fresh rat livers were homogenized on ice with four volumes of homogenization buffer (20 mM Hepes, pH 7.9, 20 mM KCl, 30% sucrose). The crude cell pellets were resuspended and lysed in 30 ml of homogenization buffer supplemented with 0.5% NP-40, and centrifuged. The nuclei were washed twice with RSB (20 mM Hepes, pH 7.9, 15% sucrose) and resuspended in one nuclear pellet volume (npv) of extraction buffer A (20 mM Hepes, pH 7.9, 5% glycerol, 200 mM NaCl). One npv of extraction buffer B (20 mM Hepes, pH 7.9, 5% glycerol, 600 mM NaCl) was added and the final NaCl concentration was adjusted to 400 mM with 5 M NaCl. The crude nuclei were extracted at 4°C for 30 minutes and the extracted nuclei were pelleted for 1 hour at 100 000 *g*. The extract was dialysed against 20 mM Hepes pH 7.9, 100 mM NaCl, 20% glycerol and 1 mM DTT, then clarified in a microcentrifuge before storage at −70°C.

Gel retardation assays. Gel retardation assays were performed essentially as described in the manual published by Ausubel et al (30).

The OL17GPA sequence for the Barbie box of the AGP gene (−142 to −126) was the following: 5′-CAGCCCAAAGCTGGCTT-3′ (the 10-bp Barbie box consensus sequence is underlined) and the sequence for NF-kB was the following: 5′-AGTTGAGGGGACTTTCCC-AGGC-3′ (the 12-bp NF-κB consensus sequence is underlined).

Reaction mixes contained 20 000-40 000 cpm of end-labeled probe, 1 μg polydI-dC and various amounts of nuclear protein in 6 μl of buffer (15 mM Hepes, pH 7.9, 4 mM Tris-base pH 7.9, 60 mM KCL, 1 mM EDTA, 1 mM DTT, 0.01% BSA, 10% glycerol). Binding reactions were run for 15 minutes at room temperature. After adding 3 μl of loading buffer, DNA–protein complexes were analyzed by electrophoresis on 7% polyacrylamide gels in Tris-glycine buffer (50 mM Tris, 380 mM glycine, 2.7 mM EDTA). Electrophoresis was run at room temperature at 200 V for 3 h in 1 x Tris-glycine. For supershift assays, nuclear protein extracts were incubated for 1 hour at 4°C with 1 or 2 μg of antiC/EBPα, antiC/EBPβ, antiNF-κB p50/52, and antiNF-κB p65 antibodies, before mixing with oligonucleotide probes.

Southwestern blot analysis. Rat liver nuclear protein extracts (60 to 80 μg) were separated on a 9% polyacrylamide-$NaDodSO_4$ gel (31). After electrophoresis, the proteins were then transferred to Westran polyvinylidene difluoride (PVDF) membranes by electrophoresis in a buffer containing 25 mM Trizma base, 190 mM glycine, 20% methanol. The sheets were blocked for four hours at room temperature with 5% (W/V) non fat dry milk in the binding buffer used for gel retardation assays. The sheets were then subjected to a binding reaction with ^{32}P-labeled oligonucleotides used in gel retardation assays (10^6 cpm/ml) at 4°C in the presence of 10 μg/ml of polydIdC and 0.5% non fat dry milk. Sixteen hours later they were washed three times for 10 min in binding buffer, dried, and subjected to autoradiography.

UV cross-linking experiments. The oligonucleotide 3′-5′ OL17GPA was synthesized with 5-Br dUTP in its sequence: 3′ GUCGGGUT-TCGACCGAA 5′, and then annealed with the oligonucleotide 5′-3′ OL17GPA. The double strand was labeled at its 5′-terminus with (γ^{32}P)ATP by using T4 polynucleotide kinase.

Labelled DNA (2 ng) was incubated with nuclear extract (25 μg) and 1 μg of polydIdc in a total volume of 40 μl as described for the gel retardation assay. The complexes were electrophoresed on 7% non denaturing gel. The gel was then irradiated with UV (254 nm) for 30 min at a distance of 10 cm from the lamp, and autoradiographed overnight at 4°C. The piece of gel with the retarded DNA–protein was excised. Gel slices were incubated overnight at 4°C in 0.2 M Tris pH 6.7, 2% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.2% bromophenol blue, before loading on SDS 9% polyacrylamide gel with validative protein markers. The gel was then dried and exposed to X-ray film at −80°C.

RESULTS

The Inductive Effect of PB on AGP Gene Expression Does Not Require Protein Synthesis

To determine whether ongoing protein synthesis was required for AGP induction by PB, we examined the effects of cycloheximide on AGP transcriptional activation by PB. SD rat hepatocytes were cultured on Matrigel and treated with PB, with or without 10 μM cycloheximide, at a concentration that produces maximal inhibition of protein synthesis (32). PB at 2 mM increased AGP mRNA content, as previously described (6), and cycloheximide treatment did not inhibit the PB effects (Fig. 1). It is unlikely that these findings reflected a lack of synthesis blockade by cycloheximide, because in PB-treated hepatocytes the increase in CYP2B1/2 mRNA content was totally abolished by cycloheximide treatment, as previously described in vivo (33-35). Thus, AGP mRNA induction by PB does not

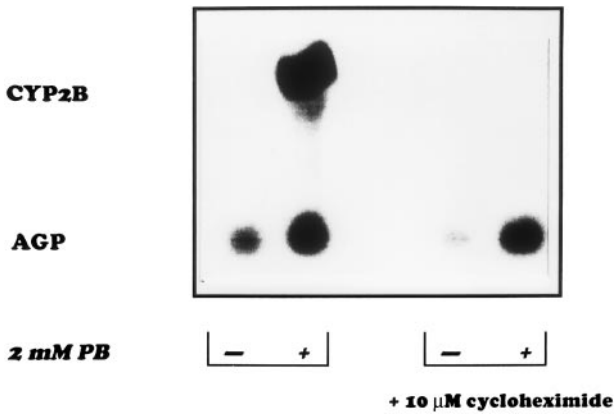

FIG. 1. Effect of cycloheximide on the induction of α1-acid glycoprotein (AGP) gene expression by 2 mM PB. Hepatocytes cultured were treated after 24 h and 48 h with PB (−/+) and 10 μM cycloheximide; controls were untreated. Total RNA was extracted 16 h after the second addition of PB and/or cycloheximide, and AGP and CYP2B mRNA were analyzed by northern blotting.

require *de novo* protein synthesis, suggesting that the increase in nuclear protein binding to the AGP Barbie box sequence in PB-treated hepatocytes is not related to an increase in protein content but rather to post-translational modifications of the corresponding proteins (mainly their phosphorylation status).

The Phosphorylation Status of Nuclear Protein Extracts Influences Their Binding Activity

To examine whether the phosphorylation status of the nuclear protein extract influenced binding to the cis Barbie box elements, nuclear extract from PB-treated and PB-untreated rat hepatocytes were incubated with calf intestinal alkaline phosphatase and shift assays were carried out with an AGP Barbie box probe. To avoid a non specific effect of the phosphatases, all nuclear protein extracts were treated in the same conditions with phosphatase, while orthovanadate, a potent inhibitor of phosphatases, was added to controls to inactivate phosphatase. Phosphatase treatment totally abolished binding both in basal and PB-induced conditions (Fig. 2), showing that the phosphorylation status of nuclear proteins governs their binding activity: dephosphorylated proteins were unable to bind to the Barbie box sequence of the AGP gene.

Participation of 43-kDa, 52-kDa, and 65-kDa Nuclear Proteins of Rat Hepatocytes in the Formation of Retarded Protein-Barbie Box Sequence Complexes

To characterize nuclear protein binding to the Barbie box sequence, nuclear protein extracts from PB-treated rats were analyzed by southwestern blotting with the OL17GPA probe, and by UV cross-linking experiments with the bromo uracil OL17GPA probe, as indicated in Materials and Methods. As shown in Fig. 3, five bands corresponding to molecular weights of 30, 43, 52, 65 and 80 kDa were detected in southwestern blots (Fig. 3A). These results, and the specificity of the bands on southwestern blots, were confirmed by cross-linking experiments. The calculated molecular weights of the bands detected by cross-linking (Fig. 3B) were 43, 52, and 65 kDa. We always observed several bands in southwestern blots experiments, while only the 43-, 52- and 65-kDa species were detected in cross-linking experiments. On the basis of these results we propose that at least three distinct proteins (43, 52, and 65 kDa) bind to the Barbie-box sequence.

The 43-kDa, 52-kDa, and 65-kDa Proteins Probably Belong to the NF-κB and C/EBP Factor Families

As their size was comparable to that of members of the NF-κB and C/EBP protein families, we examined the possibility that the 43-kDa, 52-kDa and 65-kDa protein species were NF-κB or C/EBP factors. In gel shift assays we examined changes in the binding of these proteins (contained in nuclear extracts) to the Barbie box sequence (OL17GPA probe) in competition and supershift experiments. As shown in Fig. 4, the DNA–protein interaction in PB-treated rats (lane 2 and 3) was abolished by a 100-fold molar excess of unlabeled Barbie box probe (lane 5), while the same molar excess of heterologous probe had no significant effect on binding. As shown in lane 6, the NF-κB oligonucleotide specifically and completely competed for complex formation, indicating that NF-κB- or NF-κB-related factors interact with the PBRE. Moreover, the amount of protein-DNA complexes obtained with the NF-κB probe (lane 10) was increased after PB treatment, as previously described (36), while a 100-fold molar excess of unlabelled NF-κB probe abolished the binding of the NF-κB probe to nuclear protein extracts (lane 11).

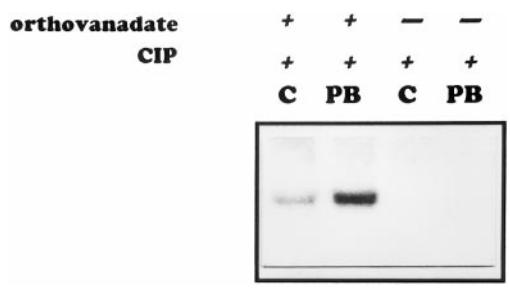

FIG. 2. Effect of phosphatase treatment on the binding of liver nuclear extracts to the labeled nucleotide representing the PBRE region of the AGP promoter. The ^{32}P-labeled probe (1 ng) was incubated with rat liver nuclear extracts (20 μg) from untreated rats (C) and rats treated with PB. Extracts were pretreated with 2 U of CIP (calf intestinal phosphatase), with or without orthovanadate (a phosphatase inhibitor).

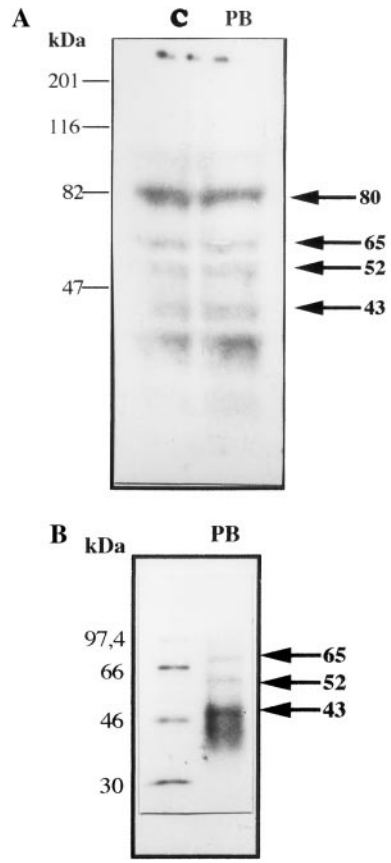

FIG. 3. (**A**) Southwestern blot of nuclear proteins extracted from control rats (C) and PB-treated (PB) rats. Proteins were resolved by SDS-PAGE. After transfer, membranes were hybridized with ^{32}P-labeled OL17GPA probe. The molecular weights of the bands were determined by comparison to a molecular weight marker. (**B**) UV cross-linking of the protein complexes formed with OL17GPA, when incubated with liver nuclear extracts from PB-treated rats. The cross-linking experiments were carried out as described in Materials and Methods. The bands were detectable after exposure of the dried gel to X-ray film at −80°C. The molecular weights of the bands were determined after correction for the contribution of single-strand OL17GPA to the total molecular weight.

The same nuclear protein extracted from PB-treated rats was then incubated with anti-p50/52 and anti-p65 NF-κB subunit antibodies before incubation with the OL17GPA or NF-κB probes. As shown in Fig. 5, 2 μg of anti-p65 (lane 5) or anti-p50/52 (lane 7) strongly reduced the binding obtained with the OL17GPA probe (lane 3). To determine the specificity of this interaction, we performed supershift assays using NF-κB as probe. One microgram of each antibody (anti-p65 or anti-p50/52) was sufficient to reduce the binding intensity (lanes 11 and 12) and to shift the bands upwards.

When supershift assays were performed with anti-C/EBPα or anti-C/EBPβ-antibodies, we also observed a decrease in the binding of nuclear proteins extracted from PB-treated rats with the OL17GPA probe and anti-C/EBPα antibodies (Fig. 6). Treatment with anti-C/EBPβ antibodies had no effect, as the intensity of binding was similar to control values.

DISCUSSION

The response to PB in many species involves transcriptional activation of numerous target genes. Many studies have focused on the molecular events regulating PB transcription of these genes, and particularly *CYP*2B1/2. The aim of the present study was to investigate the molecular mechanisms underlying the transcriptional activation of another PB target, the AGP gene. *In vivo* and *in vitro* studies in this laboratory (6,7) have led to the conclusion that the −142 to −126 sequence of the AGP promoter harbors a phenobarbital-responsive element and that the increase in nuclear protein binding to this sequence is functionally involved in the PB transactivating pathway (27).

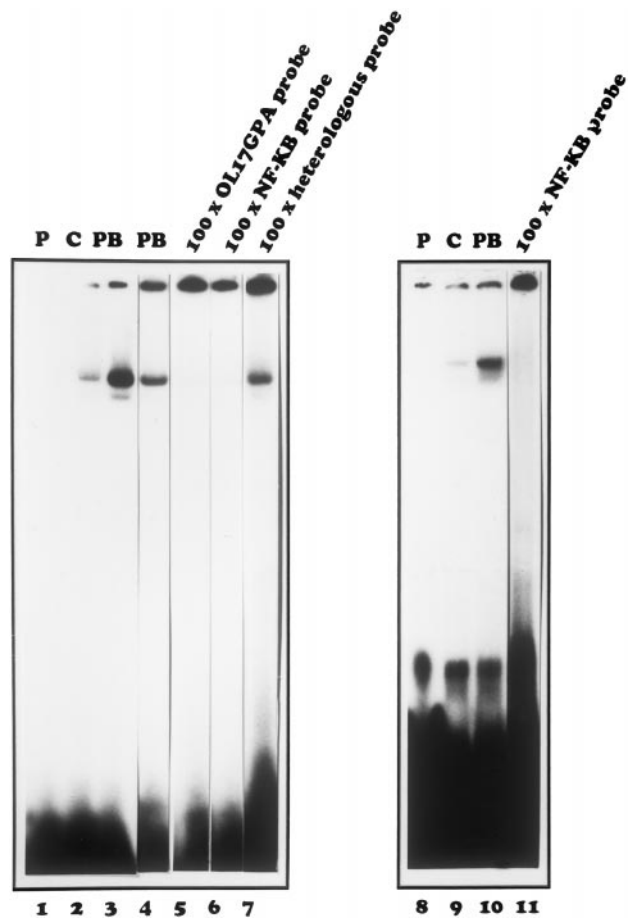

FIG. 4. Electrophoresis mobility shift assays with the labeled oligonucleotides representing either the PBRE domain (OL17GPA) (1-7) or the NF-κB consensus sequence (8-11). The ^{32}P-labeled probes were each incubated without (lanes 1 and 8, "P") or with rat liver nuclear extracts (20 μg) from control untreated (lanes 2 and 9, "c") or phenobarbital-treated rats (lanes 3, 4 and 10, "PB"). Competition analyses were performed with a 100-fold molar excess of OL17GPA (lane 5) or NF-κB (lanes 6 and 11) or heterologous probe (lane 7).

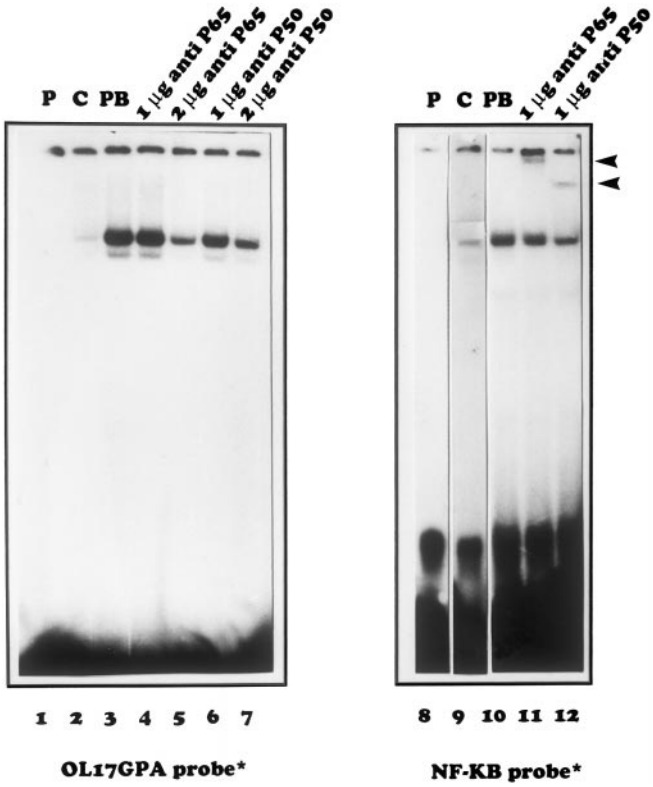

FIG. 5. Electrophoresis mobility shift assays with the labeled oligonucleotides representing either the PBRE domain (OL17GPA) or the NF-κB consensus sequence. The ^{32}P-labeled probes were each incubated without (lanes 1 and 8, "P") or with rat liver nuclear extracts (20 μg) from control untreated (lanes 2 and 9, "c") or phenobarbital-treated rats (lanes 3 and 10, "PB"). Antibody supershift assays were performed with 1 μg (lanes 4 and 11) or 2 μg (lane 5) of anti-p65 antibodies, or with 1 μg (lanes 6 and 12) or 2 μg (lane 7) of anti-p50/52 antibodies.Arrows indicate the supershifted bands.

Cycloheximide treatment of isolated and cultured rat hepatocytes showed that *de novo* protein synthesis was not required for the increase in AGP mRNA induced by PB, strongly suggesting that the nuclear proteins which bind to the PBRE sequence are constitutive and are activated post-translationally by PB. In the case of the *CYP*2B1/2 gene, conflicting observations have been reported. Burger et al (37) suggested that *de novo* protein synthesis was required in the induction of rat *CYP*2B gene expression, as in our study. In contrast, Sidhu et al (38) reported that PB induction of cytochrome P450 gene expression in rat hepatocytes did not require *de novo* protein synthesis.

Among the post-translational modifications of nuclear transmodulating protein, changes in the phosphorylation status of these target proteins (39) via cellular protein kinase and protein phosphatase activities, appear to be the major regulatory mechanisms. To assess the contribution of phosphorylation status to the capacity of nuclear protein extracted from PB-treated animals to bind to the PBRE sequence, we treated these nuclear proteins in vitro with calf intestinal alkaline phosphatase. Our results clearly show that phosphorylation status is a key regulator of the binding capacity of transactivating proteins involved in PB transcriptional activation, their dephosphorylated molecular form being unable to bind to the PBRE sequence. Several reports have recently suggested a key role of phosphorylation/dephosphorylation events in transducing the PB signal, particularly in the case of *CYP*2B1/2. However, conflicting results have again been obtained. Indeed, PB activated a protein kinase that enhanced the phosphorylation of a 26-kDa and a 94-kDa protein, which bound to a positive regulatory element of the *CYP*2B1/2 promoter (21); moreover, in primary rat hepatocytes, PB treatment resulted in transient phosphorylation of a 34-kDa nuclear protein, an event that preceded the increase in *CYP*2B1/2 mRNA accumulation (40). These results are consistent with those of Nirodi et al (35), who reported that 2-amino-purine, a general protein kinase inhibitor, had a potent inhibitory effect on PB-mediated induction of *CYP*2B1/2 gene transcription. In contrast, specific activation of the c-AMP-stimulated PKA pathway resulted in complete repression of the PB induction process in primary rat hepatocytes (41), and okadaic

FIG. 6. Electrophoresis mobility shift assays with the labeled oligonucleotide representing the PBRE domain (OL17GPA). The ^{32}P-labeled probe was incubated without (C) or with rat liver nuclear extracts (20 μg) from phenobarbital-treated rats. Antibody supershift assays were performed with 2 μg of anti-C/EBPα or 2 μg of anti-C/EBPβ antibodies.

acid, a potent and specific inhibitor of protein phosphatases, repressed the PB induction response in rat hepatocytes (42). Our results on PB-mediated activation of another PB target gene confirm that direct phosphorylation/dephosphorylation events are a key control mechanism in PB activation of several genes.

As described above, proteins of 26 kDa, 94 kDa (21) and 34 kDa (40) are involved in the DNA–protein interaction leading to transcriptional activation of *CYP*2B1/2 genes. In the case of the AGP gene, when studied by southwestern blotting and UV cross-linking experiments, we found that three common molecular weight proteins (43 kDa, 52 kDa and 65 kDa) appeared to bind to the PBRE sequence after PB treatment.

In view of the molecular weight of the proteins binding to the PBRE sequence of the AGP gene promoter, the 43-kDa species is probably C/EBP protein, while the 52-kDa and 65-kDa species probably belong to the NF-κB protein family. Our results, obtained in gel shift assays, strongly suggest the involvement of the NF-κB factor in the PBRE/protein interaction: an excess of specific probe for NF-κB protein abolished the binding, while anti-p50/52 and anti-p65 NF-κB subunit antibodies reduced it. When the AGP PBRE DNA sequence (5′-CAGCCCAAAGCTGGCTT-3′) was compared with the NF-κB sequence (5′-AGTTGAGGGGACTTTCCC-AGGC-3′) and the C/EBP consensus sequence (TTG-NNGCTAATG), the DNA sequence of PBRE does not appear to be consistent with these consensus sequences. In our view NF-κB factor and C/EBP factors may interact indirectly with the PBRE, probably via a protein/protein interaction. This is consistent with results from supershift experiments, in which anti-P50/52 and anti-P65 only weakly inhibited gel shifted complexes, probably because other interacting proteins remained bound to the probe. These other proteins are unknown, but could be related to the 30-kDa and 80-kDa species observed on southwestern blots. The involvement of NF-κB and C/EBP factors in PB induction mechanisms has already been investigated: the domains from −42 to −66 of the *CYP*2B2 gene promoter (16) and from −64 to −45 of the *CYP*2B1 gene promoter (17), which are involved in the regulation of PB induction, interact with members of the C/EBP family, particularly C/EBPα. However, no correlation between the level of C/EBPα or other C/EBP-related proteins and the induction of CYP2B1/2 genes by PB has been described. It has recently been reported that PB, in general, activates NF-κB factor in rats (36) and down-regulates its expression (43) in gel shift assays.

In conclusion, we found that the DNA–protein complex obtained with the AGP gene Barbie box involved NF-κB factors. These results are the first evidence that NF-κB factor plays a role in the transduction pathway used by PB to activate a target gene.

ACKNOWLEDGMENTS

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